BOTULINUM TOXIN TYPE A TARGETS RHOB TO INHIBIT LYSOPHOSPHATIDIC ACID-STIMULATED ACTIN REORGANIZATION AND ACETYLCHOLINE RELEASE IN PC12 CELLS: A POSSIBLE MECHANISM FOR INTERVENTION

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ABSTRACT

We showed that the G-protein activator lysophosphatidic acid (LPA) triggered actin reorganization followed by ACh release in nerve growth factor–treated PC12 cells, and that BoNT/A blocked both events through degradation of RhoB by the proteasome. Overexpression of wild-type RhoB caused to overcome toxin's inhibitory effect on actin reorganization/exocytosis stimulated by LPA. A knockdown of the RhoB gene via targeted siRNA transfection resulted in total inhibition of both actin reorganization and ACh release induced by LPA. We conclude that the RhoB signaling pathway regulates ACh release via actin cytoskeletal reorganization, and that botulinum toxin inhibits neuroexocytosis by targeting RhoB pathway.

INTRODUCTION

Neurotransmitter release is a fundamental step in synaptic transmission, and occurs via the rapid fusion of neurotransmitter-filled synaptic vesicles with the presynaptic plasma membrane (Park and Simpson, 2003). Synaptic transmission involves both vesicle trafficking and rearrangement of the actin cytoskeleton, processes that are directed by members of the Ras family of low molecular weight GTP-binding proteins (GTPases), namely Rho, Rac, and Cdc42.

The phospholipid lysophosphatidic acid (LPA) elicits a variety of biological effects in many cell types through a Rho-mediated signaling pathway (1). This actin reorganization in neuronal cells is considered to be prerequisite to neurotransmitter release from presynaptic neurons (2). In PC12 cells,

BoNTs are clostridial neurotoxins that comprise seven different serotypes, designated A-G. Botulinum toxin A (BoNT/A) inhibits ACh release at peripheral synapses by the metalloprotease activity of its light-chain (L-chain, 50 kDa) on SNAP-25 (25 kDa synaptosomal-associated membrane protein), a fusion protein essential for membrane fusion. However, deletion of SNAP-25 does not result in a complete blockade of exocytosis in NGF-differentiated (3), suggesting that there may be another pathway through which BoNT/A inhibits exocytosis.

In this study, we focused on the role of Rho GTPases and the actin cytoskeleton on regulated ACh exocytosis in NGF-differentiated PC12 cells. We show that overexpression of wild-type RhoB (wRhoB) overcomes the inhibitory effect of botulinum toxin on actin reorganization as well as ACh release stimulated by LPA. On the contrary, overexpression of a dominant negative RhoB causes inhibition of ACh release and actin reorganization by either KCl or LPA regardless of the toxin treatment.

Finally, we show that knockdown of RhoB gene expression by small interfering RNA (siRNA) results in a total blockade of ACh release and actin reorganization stimulated by LPA. We conclude that RhoB plays an important role in controlling neurotransmitter release by regulating actin cytoskeletal reorganization in PC12 cells, and that BoNT/A affects this pathway.

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MATERIALS AND METHODS

1. Antibodies and materials.

RhoA, RhoB, polyclonal antibodies, and protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Neuron-specific enolase (NSE) monoclonal antibody, proteasome inhibitors (PIs; Lactocystin, MG-132, and proteasome inhibitor I) were purchased from Calbiochem (La Jolla, CA). An enhanced chemiluminescence (ECL) reagent kit including goat anti-rabbit and mouse horseradish peroxidase–conjugated antibodies, [methyl-^{3}H]choline chloride, and PCR beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Lipofectamine 2000, and TRIzol were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum was from Hyclone (Logan, UT) and donor horse serum was from Quality Biological (Gaithersburg, MD). Nerve growth factor (NGF) was purchased from Collaborative Biomedical Products (Bedford, MA). LPA was purchased from Avanti Polar-Lipids (Alabaster, AL). Protease inhibitor cocktail, antibiotics (penicillin and streptomycin), phalloidin-tetramethylrhodamine isothiocyanate (TRITC). Botulinum toxin type A (BoNT/A) was purchased from Wako (Richmond, VA). BCA protein assay kit was purchased from Bio-Rad (Hercules, CA).

2. Cell Culture.

PC12 cells were obtained from Clontech. Cells were maintained for 4-5 days during which the medium was changed every other day (to achieve NGF-differentiated cells) at 37C in a CO_2 incubator. Cells grown to 80% confluency were exposed to 10 nM BoNT/A for 4 h.

3. ACh release study.

ACh release was measured according to the method of Ray et al., using a custom-designed perfusion chamber as described elsewhere (Ray et al., 1993).

4. Actin localization and reorganization.

Actin architecture was visualized by immunohistochemistry as described (Trifaro et al. 1993), with some modifications. Specimens were mounted with the anti-fade reagent ProLong and maintained overnight at 4°C. Immunocomplexes (actin filaments) were examined by Bio-Rad confocal and/or Nikon fluorescence microscopy imaging systems.

5. Western blotting.

To analyze the expression levels of RhoA, RhoB, and NSE, cells grown on plastic 6-well tissue culture plates were solubilized in the same lysis buffer and analyzed by western blotting using appropriate antibodies as indicated in the figure legends.

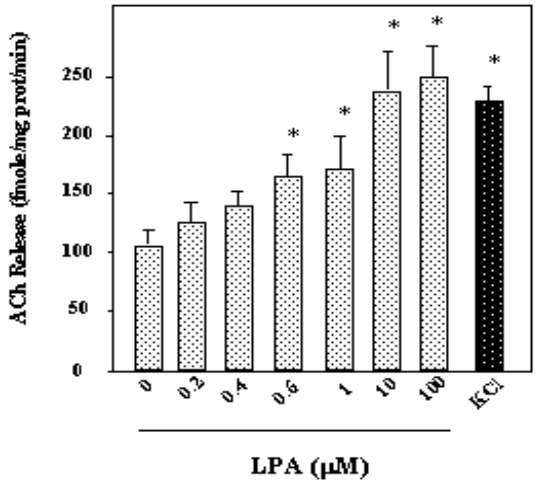
6. RNA interference. Qiagen-Xeragon (Germantown, MA) chemically synthesized the sense and antisense strands for two 21-nucleotide regions of the rat RhoB gene (5'-CAAUGUGCCCAUCAUCUUGGU-3' and 5'-AAAAAAGACCUGCGCAGCGAU-3'), 252 and 324 nucleotides downstream of the start codon, respectively. Transfection was carried out according to the manufacturer's protocol using Oligofectamine (Invitrogen). Twenty-four hours after transfection, the cells were subjected to a ACh release study, and western blotting analysis.

RESULTS

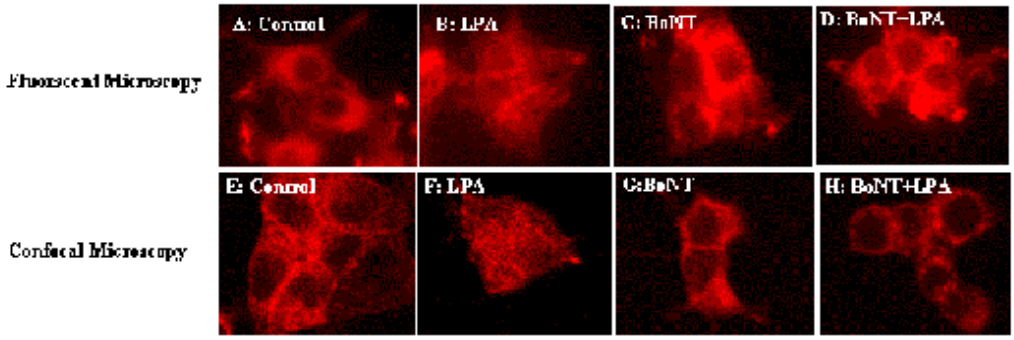
1. ACh release by LPA (Fig. 1).

We first studied whether LPA stimulates ACh release in NGF-differentiated PC12 cells by exposing cells to LPA. LPA stimulated ACh release ~1.5- to 2.5-fold over the basal release rate (no LPA). The maximum amount of ACh release by LPA was equivalent to levels (physiologically triggered by high K^+)

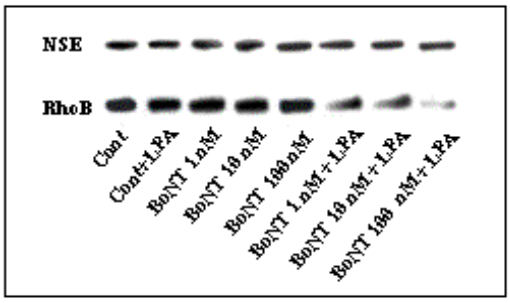
known to trigger depolarization-induced exocytosis. These data suggest that exocytosis in NGF-differentiated PC12 cells may be regulated by an LPA-stimulated G-protein signaling pathway.



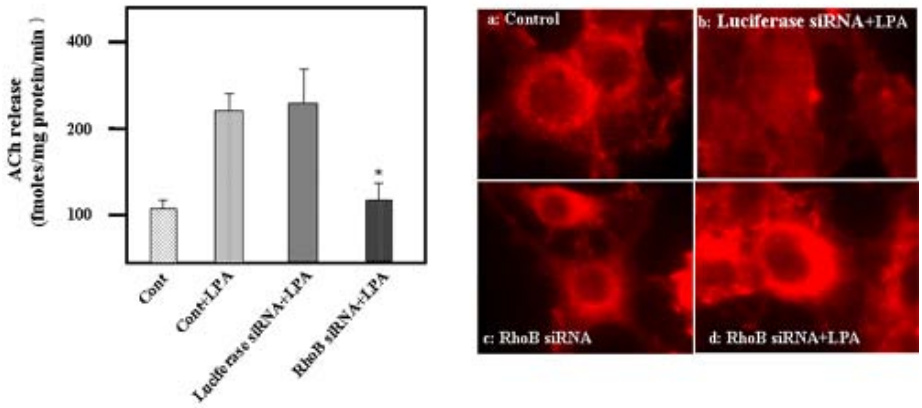
2. Effect of BoNT/LPA on Actin Architecture (Fig. 2).
Actin reorganization by LPA and the stabilization of actin architecture by BoNT/A. Fluorescence (FM) and confocal microscopy (CM) reveals actin architecture before and after treatment of cells with LPA and/or BoNT/A. In brief, PC12 cells grown on plastic slides were fixed with paraformaldehyde and permeabilized with 0.2% Triton X-100, and then incubated with phalloidin-TRITC for actin staining. Immuno-stained actin filaments were visualized using Bio-Rad confocal and/or a Nikon fluorescence microscopy systems. For the confocal images, the focal plane was set to the middle of the cells. (A, B, C, D: FM images; E, F, G, H: CM images.) (A, E) Control cells showing intense actin rings along the cell membranes, with sparse actin filament distribution in cell bodies. LPA treatment (10 M for 10 min) causes the disappearance of the actin rings, and accumulation of actin filaments in cell bodies (B, F), which did not occur after treatment with either BoNT/A (10 nM for 24 h) alone (C, G) or BoNT/A+LPA (10 μM for 10 min) (D, H), indicating the inhibitory effect of BoNT/A on actin reorganization triggered by LPA.



3. RhoB Degradation associated with LPA and BoNT (Fig. 3).
Fig. 3 show s the Western blotting analysis. Although Rhos are key regulators of the actin cytoskeleton in various cells, however, it is not known which Rho, regulates actin filament assembly involved in ACh release in PC12 cell. We used neuron specific enolase as a internal control. As shown in this Western Blotting, these are the treatments, i.e., Control, LPA, BoNT treatments with 3 different concentrations. And here, cells were first exposed to BoNT for overnight, and then stimulated with LPA. As shown here, NSE levels did not change with any treatment. RhoA levels also remain unchanged with any treatment. RhoB has more than 90% similarity in amino acid sequence with RhoA, however, a marked dose dependent decrease in RhoB immunoreactivity was evident in cells treated with BoNT/A followed by LPA stimulation.



4. Effect or knockdown of RhoB on ACh Release and Actin Reorganization (Fig. 4).
Finally, we investigated the effect of the knockdown of RhoB gene on ACh release and actin reorganization by LPA. As shown in these figures and as we expected, the gene knockdown of RhoB by transfection of the siRhoB causes total inhibition of ACh release by LPA. We can see intense actin rings in the control, with no transfection, and cells transected with siRNA for Luciferase. However, as can be seen in this picture, The cells transfected with RhoB-specific siRNAs did not undergo actin reorganization regardless of LPA stimulation.



DISCUSSION

We demonstrated Ca^{2+}-dependent ACh release (Fig. 1) from NGF-treated PC12 cells in response to LPA stimulation at levels similar to that in human serum (~10 M) (Muller et al., 2000). The magnitude of ACh release was equivalent to that initiated by a depolarizing level of KCl (80 mM; Figs. 1A, B) which induces ACh release in NGF-treated PC12 cells (4). LA is a pleiotropic phospholipid growth factor that activates the same signal transduction mechanism that is activated by polypeptide growth factors via specific G protein-coupled receptors that subsequently activate small GTPases. Our data on LPA-stimulated ACh release from NGF-treated PC12 cells indicate that the release is controlled by the G-protein-related signal transduction as well.Since exocytosis is dependent on cytoskeletal components, we hypothesized that LPA stimulates ACh release in NGF-differentiated PC12 cells by inducing actin reorganization, a prerequisite for exocytosis. As shown in Figure 2, intense actin staining in rings and sparse distribution in the cytosol was observed in untreated control cells under both FM and CM. Microscopy revealed the rapid disappearance of the ring and redistribution of actin in the cell body following the addition of LPA to the culture medium, indicating that LPA induces the reorganization of the actin cytoskeleton. Similar actin reorganization was observed when cells were treated with KCl (data not shown), again suggesting that KCl and LPA may trigger exocytosis via the same pathway.
Rhos are key regulators of the actin cytoskeleton in various cells (5). However, it is not known which Rho(s) regulates actin filament assembly involved in PC12 cell exocytosis. Since RhoB is inducible as well as degradable we tested whether treatment with BoNT/A and/or LPA affects the level of RhoB using western blotting. RhoB levels were not altered when cells were treated with either 10 M LPA for 5 min or BoNT/A (1 to 100 nM) for 4 h (Fig. 3). However, a marked dose-dependent decrease in RhoB immunoreactivity was evident in cells treated with BoNT/A followed by LPA stimulation for 5 min (Fig. 3). No such difference in immunoreactivity was evident for neuron-specific enolase (NSE, internal control) suggesting that the decrease in RhoB was not due to a general decrease in protein levels (Fig. 3).
RNA interference. To establish the role that RhoB plays in ACh release and actin reorganization, we used RNAi to deplete NGF-differentiated PC12 cells of endogenous RhoB.The effect of RhoB knockdown on ACh release and actin reorganization stimulated by LPA was studied at 24 h post-transfection of the two targeted siRNA constructs. Transfection with RhoB-specific siRNAs inhibited ACh release by LPA while transfection with the luciferase duplex had no effect (Fig. 4). Similarly, LPA-induced actin reorganization was also blocked by transfection with RhoB-specific siRNAs, as brightly stained actin rings were evident along the cell membrane regardless of LPA treatment (Fig. 9B [lower panels]). However, LPA-induced actin reorganization was not affected in cells transfected with the luciferase siRNA (Fig. 4). Given that exocytosis in all cells is absolutely dependent on actin reorganization, these results suggest that RhoB plays a critical role in neuroexocytosis in PC12 cells.

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